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Cultivating duckweed *Lemna minor* in urine and treated domestic wastewater for simultaneous biomass production and removal of nutrients and antimicrobials

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ABSTRACT

Duckweed *Lemna minor* was cultivated in human urine (HU) and the effect of urine type, dilution factor, temperature, existence of macro- and microelements on growth rate was investigated. The simultaneous removal of nutrients and selected antimicrobials was also studied in experiments with HU and treated domestic wastewater, while the starch and protein content of biomass was determined. Higher growth rates were observed at 24 °C, using HU stored for 1 d and with dilution factor equal to 1:200. In experiments with HU and wastewater, the removal of COD, total phosphorus and total nitrogen exceeded 80%, 90% and 50%, respectively, while ciprofloxacin and sulfamethoxazole were eliminated by more than 80%. The main removal mechanism for the former antimicrobial was photodegradation, while plant uptake and biodegradation seem to be of significant importance for the latter. Crude protein content reached 31.6% in experiments with HU and biomass harvesting, while starch content was enhanced when duckweed was transferred to water for 21 d, reaching 47.1%.

1. Introduction

Constructed wetland technology is a promising alternative treatment process for removing conventional and non-conventional pollutants from wastewater (Stefanakis et al., 2011; Avila et al., 2014). Among different plant-based systems, duckweed ponds are of special interest as they achieve significant removal of major pollutants and heavy metals (Sekomo et al., 2012; Zhang et al., 2014). Recent studies have also reported the removal of emerging contaminants such as pharmaceuticals and personal care products in these systems due to several biotic and abiotic mechanisms (Reinhold et al., 2010; Zhang et al., 2014). Additionally, duckweeds can produce biomass with high crude protein content due to their ability to metabolize ammonia directly from water body (Mohedano et al., 2012), while they can accumulate high percentages of starch, a fact that allow their use for bioethanol production (Xu et al., 2011; Ge et al., 2012).

In domestic wastewater, 85% of total N and 50% of total P originate from human urine (HU), indicating that separately collected HU could be used for nutrients recovery and crop production (Liu et al., 2013; Zhang et al., 2013). When urine leaves the human body it contains urea, inorganic ions, natural organic metabolites as well as traces of antimicrobials and other synthetic organic chemicals that are related to health protection and human habits. Nonetheless, literature data for urine composition vary widely; the main characteristics of HU are: pH 5–8, urea 5000–9000 mg L^{-1}, NH_4^+-N 250–8100 mg L^{-1}, COD 8000–10,000 mg L^{-1}, K^+ 1300–3100 mg L^{-1} and TP 350–2000 mg L^{-1} (Chang et al., 2013; Tuantet et al., 2014a; Zhang et al., 2013). It is worth mentioning that urine composition changes during transportation and storage, leading to an increase of pH and NH_4^+-N due to hydrolysis and a decrease of Mg due to precipitation of crystals. Regarding antimicrobials, parent compounds as well as their metabolites have been detected in HU at concentrations ranging up to some hundreds mg L^{-1}, depending on medical treatment (Gika et al., 2010; Cazola-Reyes et al., 2014).

In some recent studies, HU has been used for cultivating aquatic microorganisms in order to produce biomass that can be valorized as biofertilizer, biochemicals and biofuels. Tuantet et al. (2014a) studied the growth of *Chlorella sorokiniana* using different types of urine and in the presence of additional trace elements. Moreover, they achieved continuous cultivation of these microalgae, producing biomass that contained up to 53% w/w and 25% w/w proteins and total fatty acids, respectively (Tuantet et al., 2014b). In another study, Zhang et al. (2014) used fresh urine to cultivate *Chlorella sorokiniana*, recovering in biomass 80.4% and 96.6% of N and P, respectively; while Chang et al. (2013) reported cultivation of

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Spiroulina platensis in HU under autotrophic and mixotrophic conditions, achieving significant NH_4^+-N, P and urea removal as well as high protein content. On the other hand, there is no information for the cultivation of duckweed using HU, as well as for the characteristics of produced biomass and the removal of nutrients and antimicrobials in such systems.

Based on the above, the main objective of this study was to investigate duckweed's *Lemna minor* growth using HU. Experiments were conducted using different types of urine (fresh, hydrolyzed, stored, and synthetic) and the effect of several parameters such as urine dilution, temperature, existence of macro- and microelements on growth rate was investigated. The efficiency of *Lemna minor* to remove nutrients (COD, total N, NH_4^+-N, total P) and selected antimicrobials (sulfamethoxazole, SMX and ciprofloxacin, CIP) from HU and treated domestic wastewater was also studied; while the content of produced biomass on protein and starch was determined.

2. Methods

2.1. Chemicals and culture

Analytical standards of SMX and CIP hydrochloride were purchased from Sigma–Aldrich (Steinheim, Germany). The physicochemical properties of two selected antimicrobials can be found in Table S1. Stock solutions were prepared in methanol (Fisher, USA). Culture of *Lemna minor L.*, clone St. was donated by Federal Environment Agency (Berlin, Germany). Before their use in urine and wastewater experiments, the duckweed cultures were grown for 4 weeks in Swedish standard (SIS) sterile growth medium (Table S2) according to the conditions described by OECD Guideline 221 (OECD, 2006). All salts used for *Lemna minor* growth medium were purchased by Fluka (Heidelberg, Germany). HPLC grade water was prepared in the laboratory using a MilliQ/MilliRO Millipore system (Bedford, USA). Regenerated Cellulose (RC) filters (0.2 μm, 4 mm) for antimicrobials analysis were purchased from Phenomenex (Torrance, CA, USA). HU and secondary treated wastewater used in this study were collected from the University Campus (Lesvos island, Greece).

2.2. Experiments with Lemna minor

2.2.1. Role of different parameters on Lemna minor growth rate

Experiments were initially conducted to investigate the optimal conditions for cultivating *Lemna minor* in urine. Different dilution factors (1:2, 1:5, 1:10, 1:25, 1:50, 1:100, 1:150, 1:200, 1:250) of HU and synthetic urine (SU) were tested and the growth rates of *Lemna minor* were calculated. HU was used in three different forms (fresh, hydrolyzed, stored for 1 day at 4 °C), while not hydrolyzed SU was prepared according to Table S3. Hydrolysis of HU was achieved by continuous mixing on a shaker for 30 min at 30 °C (Tuantet et al., 2014a). Experiments were also performed at different temperatures (12 °C, 18 °C, 24 °C and 30 °C), different initial mass of duckweed (0.5 g, 1.0 g and 1.5 g) and in the presence of different macroelements (Fe, Ca, Mg) and mixture of microelements (B, Mn, Mo, Zn, Cu, Co). The experimental conditions used in each experiment are reported in Table 1.

All experiments were conducted in triplicate in glass Petri dishes (12 cm diameter), containing 100 mL of each tested media. Each Petri dish was inoculated with 12 healthy fronds of *Lemna minor* or appropriate mass of duckweed and incubated in a temperature-controlled incubator under continuous illumination with fluorescent lamps. The pH was adjusted to 7, using HCl or NaOH.

2.2.2. Nutrients and antimicrobials removal in Lemna minor experiments with urine and wastewater

Experiments with SIS medium, HU and secondary treated domestic wastewater were conducted in Petri dishes to investigate the elimination of COD, urea, NH_4^+-N, TN and TP and the removal of two antimicrobials from different classes commonly found in HU (SMX and CIP) in the presence of *Lemna minor* (Table 2). The substances were chosen according to previous studies as two of the most often used antimicrobials in Greece (Iatrou et al., 2014) that are not totally removed during conventional wastewater treatment (Thomaidi et al., 2015). The duration of the experiments was 14 days and the tested concentration for antimicrobials was 50 $\mu g\,L^{-1}$. Before the addition of target antimicrobials, toxicity tests were conducted for a wide range of concentrations (SMX: 2–2000 $\mu g\,L^{-1}$; CIP: 50–450 $\mu g\,L^{-1}$) to investigate possible toxicity of these compounds to *Lemna minor*.

Aqueous samples for the determination of nutrients and antimicrobials were taken at different time intervals, while biomass samples were taken at the beginning and at the end of the experiment to characterize duckweed for crude protein and starch content. To investigate the role of biomass harvesting on removal of nutrients and antimicrobials, experiments were also conducted with HU and harvesting of 0.5 g biomass at Days 5 and 10. To study the role of abiotic factors on the removal of antimicrobials, additional experiments were conducted in the absence of duckweed for all tested media (Table 2).

Table 1
Experimental protocol applied in *Lemna minor* growth rate experiments (number of replicates: 3).

Experiment	Type of urine	Dilution factor	Temperature (°C)	Initial number of leafs/ initial mass of duckweed (g)	pH	Duration (d)	Macro-, micro elements
A	Fresh HU	1:2, 1:5, 1:10, 1:25, 1:50, 1:100, 1:150, 1:200, 1:250	24	12 leafs	7	7	No addition
	Hydrolyzed HU, Stored HU, SU	1:150, 1:200, 1:250					
B	Stored HU, SU	1:200	12, 18, 24, 30	12 leafs	7	7	No addition
C	Stored HU	1:200	24	0.5 g, 1 g, 1.5 g	7	7	No addition
D	Stored HU	1:200	24	0.5 g	7	10	Fe[a] Ca[b] Mg[c] B, Mn, Mo, Zn, Cu, Co[d]

[a] 0.17 $mg\,L^{-1}$.
[b] 9.8 $mg\,L^{-1}$.
[c] 7.4 $mg\,L^{-1}$.
[d] B: 0.17 $mg\,L^{-1}$, Mn: 0.056 $mg\,L^{-1}$, Mo: 0.004 $mg\,L^{-1}$, Zn: 0.011 $mg\,L^{-1}$, Cu: 0.013 $mg\,L^{-1}$, Co: 0.002 $mg\,L^{-1}$.

Table 2
Experimental protocol applied in *Lemna minor* experiments investigating nutrients and antimicrobials elimination (T: 24 °C; pH: 7; Duration: 14 d).

Experiment	Growth medium	Initial mass of Lemna minor (g)	Harvesting	Antimicrobials (μg L^{-1})
A[a]	SIS medium	1.5	No	50
B[b]	Stored HU (dilution 1:200)	1.5	No	50
C[b]	Stored HU (dilution 1:200)	1.5	Yes	50
D[b]	Treated wastewater	1.5	No	50
E	SIS medium	No addition of duckweed	No	50
F	Stored HU (dilution 1:200)	No addition of duckweed	No	50
G	Treated wastewater	No addition of duckweed	No	50

[a] *Lemna minor* acclimatized in medium SIS.
[b] *Lemna minor* acclimatized to secondary treated domestic wastewater (acclimatization was conducted gradually in a period of 2 weeks).

2.2.3. *Starch accumulation in* Lemna minor *experiments with urine and wastewater*

To study starch accumulation in duckweed, duplicate experiments were conducted using SIS medium, stored HU (dilution factor: 1:200) and secondary treated wastewater in Petri dishes containing 100 mL of tested media, temperature of 24 °C, pH 7 and initial mass of duckweed equal to 1.5 g. The total duration of these experiments was 28 days and the concentration of starch was determined at Days 0, 7, 14, 21 and 28. As it has been reported in the literature that the starch content of duckweed may increase after its transfer in water containing no nutrients (Xu et al., 2011; Ge et al., 2012; Xiao et al., 2013), additional experiments were conducted with the aforementioned media. In these cases, 7 days after the start of the experiment, the cultures were transferred in Petri dishes with tap water and kept there up to the end of the experiments.

2.3. *Analytical methods*

The determination of COD, NO_3^--N, TP and TN in aqueous samples was conducted according to Standard Methods (APHA-AWWA-WPCF, 2005). The urea determination was based to the modified diacetylmonoxime colorimetric assay (Mulvenna and Savidge, 1992; Rozet et al., 2007), while an Ion Chromatography system (ICS-3000, Dionex Co., Sunnyvale, CA, USA) with suppressed conductivity detection was used for the determination of NH_4^+-N and other cations (Na, K, Mg, Ca) for the characterization of HU composition. Prior to Ion Chromatography (IC) analysis, all samples were filtered (0.45 μm) and acidified for proper preservation. Starch content in duckweed samples was determined according to anthrone method (Hansen and Møller, 1975), while calculation of crude protein was based on the measurement of TN concentration in biomass (Xiao et al., 2013). Before the determination of starch and crude protein, the fresh biomass was dried overnight at 95 °C.

For the determination of target antimicrobials, aqueous samples were filtered through RC filters (0.2 μm, 4 mm), mixed with MeOH and analyzed in an HPLC system associated with a diode-array detector (DAD) (LC-20AD/SPD-M20A/CTO-20A/SIL-20A Shimadzu, Japan). Antimicrobials were separated from medium components using isocratic separation with aqueous 0.5% HCOOH in 0.05 M CH_3COONH_4:MeOH (70:30, v/v) at a flow rate of 1 mL min^{-1}. Chromatographic separation was achieved with a Zorbax reverse phase SB-C18 analytical column (150 × 4.6 mm; 5 μm, Agilent) at 30 °C, using a guard column SB-C18. The acquisition wavelengths were 280 nm for CIP and 270 nm for SMX. The analytical procedure was based on a previously published method (Ašperger et al., 2009); the method limit of detection (LOD) was 364 ng L^{-1} for SMX and 1296 ng L^{-1} for CIP.

2.4. *Calculations and data analysis*

For the comparison of *Lemna minor* culture growth under different conditions, the specific growth rate in each condition was calculated against the culture grown in SIS medium (control). Specific growth rate was calculated by a linear regression of the natural logarithm versus culturing time, according to the following equation (OECD, 2006; Gatidou et al., 2015):

$$\mu_{(i-j)} = \frac{\ln N_j - \ln N_i}{t} \quad (1)$$

where, $\mu(i–j)$ is the average growth rate from time i to j, N_i and N_j are the corresponding biomass amount (g) or leaf number and t is the time period from i to j.

Specific growth rate normalized to area (μ_{area} as g m^{-2} d^{-1}) was calculated according to Eq. (2) using fresh or dry weight mass data (Zhao et al., 2014; Xiao et al., 2013).

$$\mu_{area} = \frac{IW}{A \times t} \quad (2)$$

where, IW is the average increased weight of dry or fresh biomass, A is the area of Petri dish and t is the total time period of the experiment.

OriginPro 8 SR0 (Version 8.0724, OriginLab Corporation, Northampton, USA) was used for the construction of all graphs in current study. For the selection of best cultivation parameters, growth rates were checked with SPSS 21.0 by one-way ANOVA for the role of temperature and with two-way ANOVA for the type of urine, the dilution rate as well as of the effect of the initial mass of *Lemna minor* in relation to the type of the substrate. A three-way ANOVA was used to examine the effects of time, type of substrate and the transfer of the cultures in water containing no nutrients. When ANOVA was significant at $p < 0.05$, the Tukey's HSD post hoc test was run to identify differences between treatments.

3. Results and discussion

3.1. *Role of different factors on* Lemna minor *growth*

Experiments were conducted to investigate *Lemna minor* growth in urine. The characteristics of fresh HU are presented in Table 3 and were comparable to the values commonly found in the literature. According to leaf measurements, a growth rate of 0.33 d^{-1} was calculated for *Lemna minor* cultivated in SIS medium, whereas when urine was used the higher growth rates (0.20–0.24 d^{-1}) were observed for HU that had been diluted 200 times before the experiment (Fig. 1). When smaller dilution rates were applied, a significant inhibition of duckweed growth ($p < 0.05$) was observed, ranging from 42% to 97% for dilution factors of 1:150–1:2, respectively. For dilution factor equal to 1:200, comparison among different types of HU types showed that the higher growth and the best characteristics of *Lemna minor* leafs (green and healthy) were observed for urine that had been stored for 1 d before use as well as for SU; slower growth and pallid leafs were observed in experiments with hydrolyzed HU. It is widely known that during hydrolysis urea is hydrolyzed by the enzyme urease to ammonia and carbamate (Udert et al., 2003; Liu et al., 2013; Tuantet et al., 2014a). The high concentrations of ammonia in hydrolyzed urine observed in this study (Table 3) could affect *Lemna minor* growth

Table 3
Characteristics of fresh, hydrolyzed and stored human urine (HU) used in this study and typical synthesis for fresh HU reported in the literature.

Parameter	Fresh HU[a]	Fresh HU[b]	Hydrolyzed HU[a]	Stored HU[a]
pH	6.4 ± 0.1	5.0–8.0	8.5 ± 0.3	7.0 ± 0.1
Conductivity, mS cm^{-1}	10.0 ± 0.2	6.0–23.0	19.0 ± 0.1	11 ± 0.1
Urea, mg L^{-1}	8000 ± 200	5000–9000	3660 ± 200	8000 ± 200
TP, mg L^{-1}	1020 ± 57	350–2000	800 ± 20	1100 ± 20
TN, mg L^{-1}	6000 ± 120	4000–10,000	5400 ± 80	4200 ± 120
NO_3^-N, mg L^{-1}	280 ± 11	–	–	–
NH_4^+-N, mg L^{-1}	486 ± 16	250–8100	1276 ± 60	572 ± 20
COD, mg L^{-1}	9000 ± 155	8000–15000	4000 ± 200	8000 ± 160
Na, mg L^{-1}	3000 ± 90	1800–5800	3200 ± 20	3000 ± 40
K, mg L^{-1}	3040 ± 14	1300–3100	2000 ± 18	3000 ± 16
Mg, mg L^{-1}	186 ± 8	29–121	24 ± 4	60 ± 4
Ca, mg L^{-1}	252 ± 11	96–233	300 ± 80	220 ± 8

[a] Data from current study.
[b] Chang et al. (2013); Zhang et al. (2013); Tuantet et al. (2014a,b).

(Tuantet et al., 2014a; Xiao et al., 2013). HU stored for 1 d with dilution 1:200 were selected as the best medium for the following experiments with the highest growth rate ($p < 0.01$).

To investigate the role of temperature on duckweed growth, experiments were conducted at four different temperatures in SIS, stored and synthetic HU. The two-way ANOVA showed that both temperature and type of substrate significantly affected the duckweed growth ($p < 0.05$), while their interaction was not significant. Specifically, for stored and synthetic HU, the highest growth rate was observed at 24 °C (Fig. 2) with significant statistical differences ($p < 0.001$). In experiments with stored HU and SU at temperature of 18 °C, the growth rate decreased by 40% comparing to 24 °C, while inhibition higher than 85% was noticed at temperatures of 12 °C and 30 °C. According to the protocol describing the use of *Lemna minor* for toxicity tests (OECD, 2006), this organism can be maintained at lower temperatures (4–10 °C); however the running of experiments at 24 °C is proposed. Moreover, greater growth rates of duckweeds at temperatures ranging between 22.5 °C and 27.5 °C have been reported by Xiao et al. (2013).

The role of micronutrients and macronutrients on duckweed growth was investigated in experiments conducted with stored HU and dilution 1:200 as described in Table 1. No improvement on *Lemna minor* growth rate was noticed in the presence of micro/macronutrients (Fig. S1). In a previous study (Xiao et al., 2013), the addition of micronutrients increased growth rate of different duckweed species (*Spirodela polyrhiza* (L.), *Lemna aequinoctialis* P1, *Landoltia punctata* S3, *La. punctata* OT); whereas to the best of our knowledge no data is available for the role of micronutrients or added macronutrients on *Lemna minor* growth.

The role of initial duckweed mass on growth rate was investigated using HU and SIS medium. Three different initial masses of *Lemna minor* (0.5 g, 1.0 g, 1.5 g) were tested and the specific growth rate, μ, as well as specific growth rate normalized to area, μ_{area}, were calculated (Table 4). According to the statistical analysis, both the initial mass of duckweed and the type of substrate affected μ and μ_{area} ($p < 0.001$). Specifically, the highest growth rates were observed at initial mass of duckweed equal to 1.5 g, while under these experimental conditions, both μ and μ_{area} were higher in HU

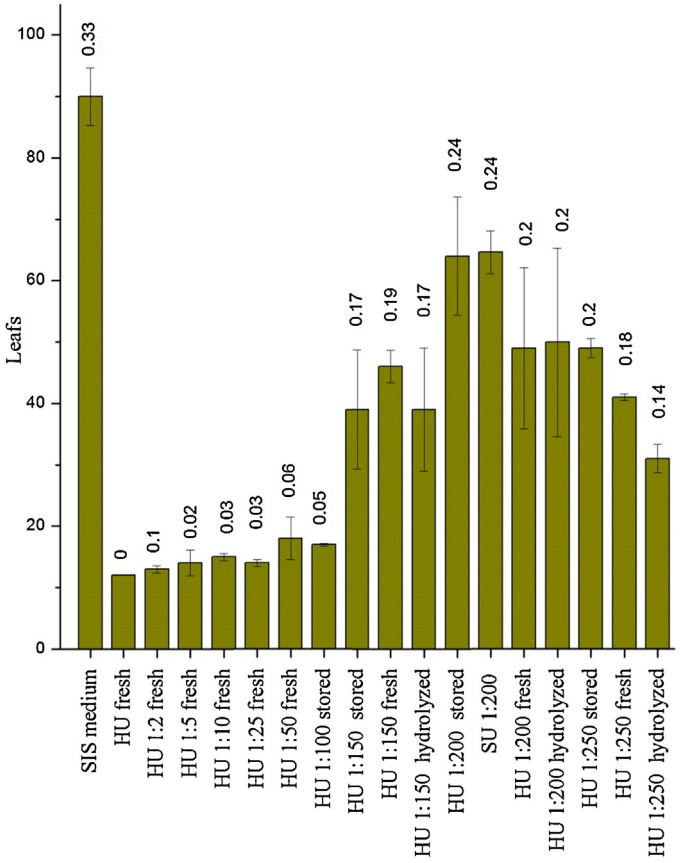

Fig. 1. Effect of urine type and dilution factor on the number of leafs and growth rate, μ (as d^{-1}) of *Lemna minor*. The values above each column represent calculated growth rates (duration of the experiment: 7 d; 12 leafs initial frond number; temperature: 24 °C; pH 7; growth rates were calculated using Eq. (1)).

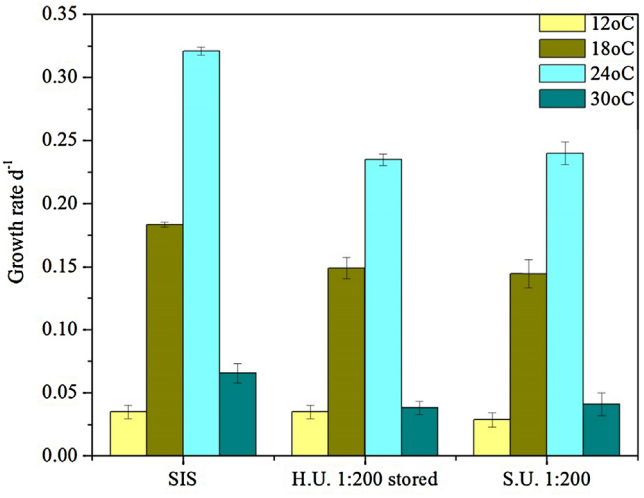

Fig. 2. Growth rate values, μ (d^{-1}) from leaf measurements for tested temperatures in stored human urine (HU) and synthetic urine (SU) compared with control medium SIS (duration: 7 d; 12 leafs initial frond number; temperature: 24 °C; pH adjust to 7; growth rates were calculated using Eq. (1)).

Table 4
Role of initial amount of *Lemna minor* on the production of biomass (g), specific growth rate, μ (d^{-1}) and specific growth rate normalized to area, μ_{area} (duration of the experiment: 14 d; temperature: 24 °C; pH: 7).

Parameters	Initial Amount of *Lemna minor* (g)		
	0.5	1.0	1.5
SIS Medium			
Duckweed mass (g) (Day 14)	1.41 ± 0.05	2.88 ± 0.1	4.73 ± 0.4
Growth rate, μ (d^{-1})[a]	$0.074^{a,A}$ ± 0.003	$0.076^{a,A}$ ± 0.003	$0.082^{b,A}$ ± 0.006
Growth rate, μ_{area} ($g\,m^{-2}\,d^{-1}$)[b]	$0.089^{a,A}$ ± 0.003	$0.182^{b,A}$ ± 0.007	$0.299^{c,A}$ ± 0.026
Human urine			
Duckweed mass (g) (Day 14)	1.60 ± 0.05	3.60 ± 0.1	5.83 ± 0.35
Growth rate, μ (d^{-1})[a]	$0.083^{a,B}$ ± 0.002	$0.091^{a,B}$ ± 0.002	$0.097^{b,B}$ ± 0.04
Growth rate, μ_{area} ($g\,m^{-2}\,d^{-1}$)[b]	$0.101^{a,B}$ ± 0.003	$0.227^{b,B}$ ± 0.006	$0.369^{c,B}$ ± 0.022

Means sharing the same superscript are not significantly different at the .05 level with respect to the initial mass of duckweed (a–c) and to the type of substrate (A–B).
[a] Calculated according to Eq. (1) and using data of dry biomass.
[b] Calculated according to Eq. (2).

than in the control experiment with SIS medium. For that reason, the initial mass of 1.5 g was chosen for the following experiments. It should be mentioned that the selected plant density mimicked the full surface coverage growth observed in natural and treatment wetlands (Reinhold et al., 2010). According to the literature, for a specific range of plant densities, when more fronds initially exist greater biomass production can be achieved. On the other hand, the application of very high plant densities can inhibit duckweeds growth due to overcrowding phenomena (Xu et al., 2011; Xiao et al., 2013).

3.2. *Removal of nutrients in experiments with* Lemna minor

The removal of major pollutants in experiments with stored HU and treated wastewater is presented in Fig. 3 and Table S4. According to the results, *Lemna minor* efficiently removed COD and nutrients from HU and treated wastewater. Specifically, in experiments with HU, very high (>95%) COD and TP removal was achieved up to the end of the experiment (14 d), whereas the removal of urea, TN and NH_4^+-N was higher than 83%, 50% and 55%, respectively. Similar results were found when biomass was harvested during the experiment. High nutrients removal was also observed in experiments with treated wastewater, ranging from 70% to 100% for TN and NH_4^+-N, respectively. The preference of *Lemna minor* to remove nitrogen in the form of ammonia has been reported in the literature (Ge et al., 2012; Chang et al., 2013). Concerning TP, the results of the current study are similar or even higher compared to those reported in the literature for other tested media (Xu et al., 2011; Xu and Shen, 2011).

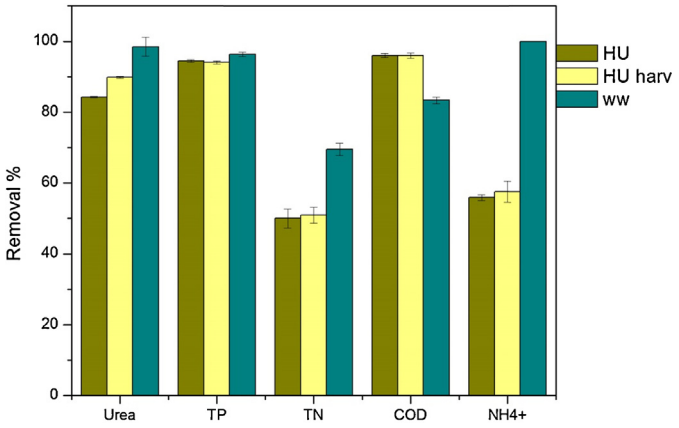

Fig. 3. Removal of urea, TP, TN, COD and NH_4^+-N in experiments with human urine (HU), human urine and harvesting of biomass (HU harv) and secondary treated wastewater (ww). (duration of the experiment: 14 d; initial mass of duckweed: 1.5 g; temperature: 24 °C; pH: 7).

The changes in concentrations of urea and NH_4^+-N during the experiment are shown in Fig. S2 and Table S4. Urea is hydrolyzed into NH_4^+-N prior its assimilation and hence the concentration of NH_4^+-N was found to increase at the beginning of the cultivation period (Fig. S2). Similar trends for the concentrations of urea and NH_4^+-N have been reported in previous studies, investigating the cultivation of *Spiroulina platensis* in HU (Chang et al., 2013).

3.3. *Removal of antimicrobials in experiments with* Lemna minor

The elimination of SMX and CIP was investigated in abiotic and biotic experiments with SIS medium, HU and secondary treated wastewater. Before adding the antimicrobials in Petri dishes with duckweed, their toxicity on *Lemna minor* was tested for a wide range of concentrations (Fig. S3). CIP greatly affected *Lemna minor* growth and leafs' characteristics (yellow leafs with chlorosis) at concentrations equal or higher than 150 $\mu g\,L^{-1}$, while no considerable effect was noticed for SMX at concentrations up to 2000 $\mu g\,L^{-1}$. Based on the above, no toxic effects on *Lemna minor* are expected for the concentrations used in the current study (50 $\mu g\,L^{-1}$).

During the 14 d of the experiment, in the absence of *Lemna minor*, SMX was removed by a factor of 10% from SIS medium and HU and by 30% in the presence of treated wastewater (Fig. 4A1). On the other hand, an almost total removal was observed for CIP under abiotic conditions up to the end of experiment (Fig. 4B1). This removal of CIP is probably due to photodegradation as according to the literature this compound is very photosensitive (Girardi et al., 2011; Babić et al., 2013). The presence of duckweed improved significantly the removal of SMX, exceeding 80% in all tested media up to the end of the experiment (Fig. 4A); whereas CIP removal was slightly decreased comparing to abiotic experiments (Fig. 4B). These results indicate the role of plant uptake and bacterial activity on removal of SMX, while the deceleration of CIP removal could be due to the prevention of light penetration in the presence of duckweed.

The aforementioned results indicate the potential efficiency of systems with duckweeds to remove antimicrobials. Further experiments should be conducted with *Lemna minor* to evaluate the role of different mechanisms such as photodegradation, hydrolysis, biodegradation and plant uptake on the removal of antimicrobials and to identify the transformation by-products.

3.4. *Starch accumulation and crude protein in* Lemna minor *experiments with urine and wastewater*

The content of *Lemna minor* in crude protein and starch at the start and at the end (14 d) of experiments with SIS medium, HU and

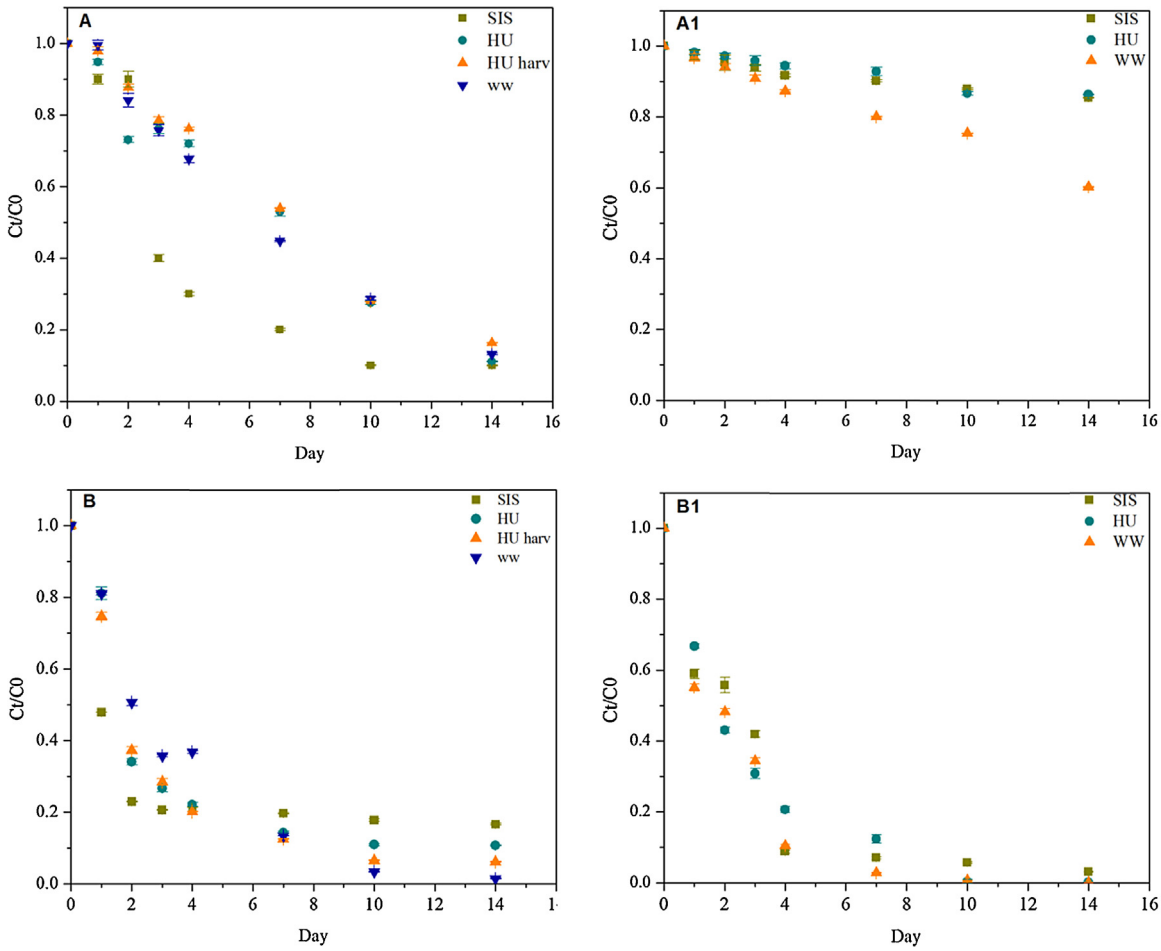

Fig. 4. Removal of sulfamethoxazole, SMX (A) and ciprofloxacin, CIP (B) in experiments with *Lemna minor* cultivated in different tested media and removal observed under abiotic conditions, SMX (A1) and CIP (B1) (duration of the experiment: 14 d; initial mass of duckweed: 1.5 g; temperature: 24 °C; pH: 7; SIS: control medium; HU: human urine; HU harv: human urine with harvested biomass at Days 5 and 10; WW: secondary treated wastewater).

Table 5
Crude protein and starch content of *Lemna minor* cultivated in SIS medium, human urine (HU) and secondary treated wastewater (duration of the experiment: 14 d; initial mass of duckweed: 1.5 g; temperature: 24 °C; pH: 7).

Days	SIS medium	Human urine	Human urine harvested	Treated wastewater
Crude protein (%)[a]				
0	21.82	25.32	25.32	25.32
14	23.29	28.76	31.60	25.26
Starch (%)[b]				
0	17.7	21.0	21.0	21.0
14	26.7	31.8	28.85	33.8

[a] Value of one sample per media.
[b] Mean value of two samples per media.

treated wastewater is shown in Table 5. According to the results, the highest protein content (31.6%) was observed in experiments with HU and harvesting of biomass, whereas the highest starch content in experiments with treated wastewater (33.8%). The levels of crude protein from current study are comparable to previous studies conducted with different media such as diluted swine and lagoon wastewater where the crude protein ranged between 10% and 40% (Mohedano et al., 2012; Xu et al., 2011; Xiao et al., 2013).

It is known that fronds are the dominant starch storage organ, while there is a negative relationship between growth rate of the duckweed and starch storage (Ge et al., 2012; Xiao et al., 2013). To achieve higher starch accumulation an additional experiment took place for 28 d in the absence and presence of nutrients, as described in Section 2.2.3. According to the statistical analysis of the results summarized in Table 6, the 'time' (that is the day of the determination), the 'type of substrate' (SIS, HU, treated wastewater) and the 'transfer' of the cultures in water containing no nutrients all exerted significant effects (at the 0.001 level) on the starch content. The post hoc tests revealed that the starch content increased significantly in all tested media, regardless the presence or absence of nutrients, up to the 21st d of the experiment ($p < 0.001$), while it remained constant up to the end of the experiment (28th d) ($p > 0.05$ between 21st and 28th d). The starch accumulation followed the order SIS < treated wastewater < HU ($p < 0.01$), whereas in the experiments conducted in the absence of nutrients (transportation of duckweed cultures in tap water at day 7), higher starch content was recorded than in treatments in media containing nutrients ($p < 0.001$) for all the examined substrates. Summing up the all

Table 6
Starch content (%) in *Lemna minor* cultivated in SIS medium, human urine and treated wastewater (duration of the experiments: 28 d; initial mass of duckweed: 1.5 g; temperature: 24 °C; pH: 7).

Sample/Day	0	7	14	21	28
SIS medium[a]	$17.9^{1,a,A} \pm 0.0$	$18.8^{2,a,A} \pm 0.1$	$26.1^{3,a,A} \pm 0.1$	$31.0^{4,a,A} \pm 0.0$	$31.4^{4,a,A} \pm 0.1$
Human urine[a]	$19.9^{1,c,A} \pm 0.1$	$24.6^{2,c,A} \pm 0.1$	$30.4^{3,c,A} \pm 0.2$	$40.1^{4,c,A} \pm 0.1$	$40.9^{4,c,A} \pm 0.1$
Wastewater[a]	$19.9^{1,b,A} \pm 0.1$	$23.9^{2,b,A} \pm 0.0$	$28.2^{3,b,A} \pm 0.2$	$37.1^{4,b,A} \pm 0.1$	$37.8^{4,b,A} \pm 0.1$
SIS medium[b]	$17.3^{1,a,B} \pm 0.1$	$19.7^{2,a,B} \pm 0.1$	$28.4^{3,a,B} \pm 0.1$	$37.3^{4,a,B} \pm 0.1$	$38.6^{4,a,B} \pm 0.1$
Human urine[b]	$19.9^{1,c,B} \pm 0.1$	$24.3^{2,c,B} \pm 0.0$	$34.3^{3,c,B} \pm 0.1$	$46.1^{4,c,B} \pm 0.0$	$47.1^{4,c,B} \pm 0.1$
Wastewater[b]	$19.9^{1,b,B} \pm 0.1$	$24.5^{2,b,B} \pm 0.1$	$31.9^{3,b,B} \pm 0.0$	$45.9^{4,b,B} \pm 0.0$	$43.4^{4,b,B} \pm 0.1$

[a] All media remained the same until the end of the experiment.
[b] Cultures were transferred in dishes with tap water 7 d after the start of the experiment. Means sharing the same superscript are not significantly different at the .05 level with respect to the day of the determination (1–4), the type of substrate (a–c) and the transfer to tapwater (A–B).

the above, it follows that the highest starch content was reached by duckweed cultivated for at least 21 d in HU after transfer to tap water at Day 7. As it has been previously mentioned, the deficiency of nutrients (N and P) helps duckweeds to faster accumulate starch (Xiao et al., 2013). The starch content achieved in this study is comparable to other studies conducted with agricultural and swine wastewater and where starch content in the range of 12.5–52.9% has been reported (Ge et al., 2012; Xu et al., 2011; Xiao et al., 2013).

4. Conclusions

Duckweed ponds could be a low-cost and eco-friendly solution for wastewater treatment and valorization. The cultivation of *Lemna minor* using HU or treated wastewater achieve significant removal of major pollutants, efficient elimination of SMX and production of biomass with high starch for possible use as biofuel. For both test media, removal of urea, COD, TP, TN, NH_4^+-N, SMX and CIP exceeded 84%, 83%, 94%, 50%, 58%, 82% and 88%, respectively. The major mechanisms governing SMX and CIP removal were plant uptake and photodegradation, respectively. The higher starch content was achieved when biomass was cultivated in HU for 7 d and then transferred to tap water for 21 d.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ecoleng.2015.09.071.

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